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(54) Title: PHYTASE FROM GERMINATED SOYBEANS

(57) Abstract

The invention relates to a class of phytate–degrading enzymes which are endogenously present in soya flour, soybeans, germinated soybeans or fractions thereof, to a method for obtaining such enzymes as well as to the use of these enzymes in feed and food applications.

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Phytase from Germinated Soybeans

The invention relates to a novel class of phytate-degrading enzymes, in particular phytases (myo-inositol hexaphosphate hydrolases), which are endogenously present in soya flour, soybeans, germinated soybeans or fractions thereof, to a method for obtaining such enzymes as well as to the use of these enzymes in feed and food technology.

Background of the Invention

Phytases are enzymes which catalyse the hydrolysis of phytic acid (myo-inositol hexakis phosphate) to myo-inositol and inorganic phosphate.

Phosphorous is an essential element for growth in animals. Animal diets contain large amounts of phosphorous, the major part being present as phytate (phytic acid) which is largely not available for monogastric animals. Since phosphorous is essential for animal metabolism and the inorganic phosphate of the phytate molecule is largely unavailable to such animals, this dietary source of phosphorous passes through the digestive tract without contributing to the nutrition of said animals and is excreted in substantial quantities in the manure. Consequently, the application of large amounts of manure to farmland for agriculture purposes leads to an accumulation of phosphorous in the soil and to environmental pollution.

To increase the nutrient availability of phytate phosphorus to animals and to decrease the environmental phosphorous load, commercially available enzyme preparations containing phytases are mixed with feedstuffs.

A second reason to degrade phytate in animal diets is due to the fact that phytate has additional anti-nutritional properties since phytates are able to form complexes with multivalent cations (Erdman, 1979) making these metals less available for absorption in the digestive tract.

Phytases are produced by micro-organisms like *Aspergillus niger* (Vollova et al., 1994), *Aspergillus ficuum* (Ullah and Dischinger, 1995), *Aspergillus carbonarius* (Al-Asheh and Duvnjak, 1994), *Klebsiella aerogenes* (Tambe et al., 1994) and *Bacillus subtilis* (Shimizu, 1992).

Phytases are also produced by plants. Sutardi and Buckle (1986) and Gibson and Ullah (1988) have partially purified and characterised a phytase from soybeans. Chang and Swimmer (1976) have characterised phytase of Phaseolus vulgaris beans. Phytases were also found in peas (Beal and Mehta, 1985), barley malt (Lee, 1990), wheat (Khare et al., 1994) and rye (Fretzdorff and Weipert, 1986).Germination of certain seeds has been found to increase phytase activity. Mandal and Biswas (1970) did not find any phytase activity in the cotyledons of ungerminated mung beans, but showed that phytase activity did appear during germination. Subsequently, they purified and characterised this phytase activity from germinated mung beans soaked for 72 hr (Mandal et al., 1972). Eskin and Wiebe (1983) examined the phytase activity of two Faba bean cultivars during germination and Houde et al. (1990) purified and characterised a phytase from 7-day germinated canola seeds.

The common sources of industrially available phytases are the fermentation broths of micro-organisms.

The use of phytases in the feed industry has become increasingly important due to their phosphorous releasing activity on phytic acid present in feedstuffs. The use of

phytase serves to increase the availability of bound phosphate and complexed multivalent cations to monogastric animals. This leads to an increased bioavalability of phytate phosphate, better utilisation of the available nutrients in the animal diets, lowered phosphorous content of the manure and, consequently, reduces the impact of livestock production on the environment.

Summary of the Invention

In view of the importance of phytases in feedstuffs, an object of the present invention is to provide a novel class of soybean phytase which has specific characteristics with respect to pH optima and temperature stability, etc., different from most microbial enzymes, and are therefore of importance for particular industrial applications.

A further object of the invention is to provide a method for obtaining and purifying phytase of the invention from soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof.

A further object of the present invention is to provide DNA molecules encoding the phytase of the present invention, prokaryotic or eukaryotic organism or host cell transformed with a DNA molecule encoding phytase of the invention and capable of expressing said phytase and a recombinant method for producing phytase from said prokaryotic or eukaryotic organisms or host cells.

A further object of the invention is to provide for the use of the phytases of the invention for foods and animal feedstuffs and for reducing the environmental impact of phosphorous from livestock production and reducing the multivalent metal ion binding antinutritional effect of phytate.

The object of the present invention is solved by making available a phytase which is obtainable by extracting soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof using an aqueous solvent and selectively precipitating proteinaceous material from the extract obtained or from a fraction thereof, and optionally further fractionating the precipitate.

Other objects will become apparent from the following detailed specification.

Subject matter of the phytase of the invention is a soybean phytase which has an optimal pH of about 5.0 when measured in a buffer comprising 0.0091 M sodium phytate in 50 mM acetic acid/NaOH and 1 mM $CaCl_2$ at 50°C for 4 hrs.

A further preferred embodiment of the phytase of the invention is that said phytase has a specific activity of at least 6 µmol/min/g protein, preferably 21 µmol/min/g protein, and more preferably, 1.3 mmol/min/g protein when measured in a buffer comprising 0.0091 M sodium phytate in 50 mM acetic acid/NaOH and 1 mM $CaCl_2$ at 50°C for 4 hrs at pH 5.0.

A further embodiment of the phytase of the present invention is that said phytase has a pI of about 4.9.

A further embodiment of the phytase of the present invention is that said phytase has a molecular weight of between 30,000 and 100,000 Daltons, preferably about 75,000 Daltons.

In a further preferred embodiment, the phytase of the invention comprises an amino acid sequence in the N-

terminal portion of the protein having at least 85% homology to the amino acid sequence given in SEQ ID NO: 1 and/or SEQ ID NO: 3, and, more preferably, comprises the amino acid sequence given in SEQ ID NO: 1 in the N-terminal portion of the protein and/or the internal amino acid sequence as given in SEQ ID NO: 3.

Subject matter of the present invention is also a purified phytase obtainable by a method comprising the steps of:

a) subjecting an extract of soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof to 50-70% ammonium sulfate precipitation to form a precipitate comprising said phytase;
b) subjecting said precipitate to anion exchange chromatography and collecting fractions containing said phytase; and
c) subjecting said precipitate to cation exchange chromatography and collecting fractions containing said phytase.

The present invention relates to phytases with one or more of the above characteristics.

The present invention also provides a DNA molecule and vector DNA molecule encoding a phytase according to the invention as well as prokaryotic or eukaryotic organism or host cell transformed with said DNA molecule and capable of expressing said phytase. Preferably said prokaryotic host cell is selected from the group comprising E. coli, Bacillus sp., Lactobacillus sp. and Lactococcus sp. Preferably said eukaryotic organism or host cell is a fungus selected from the group comprising Aspergillus, Trichoderma, Penicillium, Mucor, Kluyveromyces and Saccharomyces or is a plant selected from the group comprising soybean, corn and rapeseed and seeds thereof.

Further subject matter is a method of producing phytase of the present invention comprising the steps of:

a) subjecting an extract of soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof to 50-70% ammonium precipitation to form a precipitate comprising said phytase;

b) subjecting said precipitate to anion exchange chromatography and collecting fractions containing said phytase; and

c) subjecting said precipitate to cation exchange chromatography and collecting fractions containing said phytase.

Preferably, said anion exchange chromatography is performed using a Source Q column and/or said cation exchange chromatography is performed using a Source S column.

Further subject matter of the invention is the use of the phytase of the present invention in seeds, foods or animal feeds and for the treatment of cereals, legumes and other agro-materials for the preparation of food materials or feedstuffs or fractions thereof.

Further subject matter of the invention is the use of the phytase and organisms of the present invention, preferably plants or seeds, for reducing the environmental impact of phosphorous from livestock production and reducing the divalent metal ion binding antinutritional effect of phytate.

Brief Description of the Drawings

Figure 1: Protein content and phytase activity in fractions collected by chromatography on a Source Q column as described in Example 3.

Figure 2: Protein content and phytase activity in fractions collected by chromatography on a Source S column as described in Example 4.

Figure 3: SDS-PAGE analysis of the isolated phytase of the invention after column chromatography on Source Q and subsequent column chromatography on Source S; lane 1: molecular weight marker; lane 2: purified phytase, reduced; lane 3: purified phytase, non-reduced.

Figure 4: Isoelectric focusing of the isolated phytase of the invention after column chromatography on Source Q and subsequent column chromatography on Source S; lane 1: molecular weight marker; lane 2: purified phytase.

Figure 5: The pH profile of the phytase of the invention after column chromatography on Source Q and subsequent column chromatography on Source S.

Detailed Description of the Invention

Example 1:

Soybeans (500 g, *cv. Williams 82*, Illinois Foundation Seeds, Champaign, Il, USA) were sterilised by washing with 0.5 % (w/v) sodium hypochloride for 30 minutes. Subsequently the beans were washed five times (five minutes each time) with sterile water. The sterilised soybeans were germinated in six glass covered dishes with filter paper on the bottom (sterilised with 70% ethanol) at 20°C for 2 to 8 days in the dark. After germination, the soybeans were frozen at -24°C, lyophilised and milled in a Retch mill at 1 mm. The milled soybeans were extracted with 0.05 M acetic acid/NaOH, pH 5.0, containing 1 mM $CaCl_2$ and 0.1% Tween-20,

stirred at 20°C for 1.5 hrs, the suspension was centrifuged at 5°C at 16,000 g for 40 min and the resulting floating lipid layer was removed. The supernatant was fractionated by ammonium sulphate precipitation. The protein pellet fraction obtained after 50 to 70 % ammonium sulphate precipitation contains proteins with a specific phytase activity of 6 µmol/min/g protein.

Example 2:

The protein content was measured with the Bio-Rad Protein assay (Bio-Rad, Veenendaal, the Netherlands). 1 ml of Bio-Rad Protein assay reagent (1 part was diluted with four parts H_2O) was mixed with 25 µl of sample. After a period of 5 minutes to one hour the absorbence was measured at 595 nm. Ovalbumin was used for calibration.

The phytase activity was measured according to Simon et al. (1990). The specific phytase activity is described by the amount of phosphate which is liberated from 0.0091 M sodium phytate by 1 g protein at 50 °C and pH 5 during one minute under the conditions of the assay. The phytase activity determination was carried out in 96 well microtiter plates. 50 µl of sample was incubated with 100 µl of 0.0091 M sodium phytate in 50 mM acetic acid/NaOH, pH 5.0, containing 1 mM $CaCl_2$ at 50°C for 4 hrs. The incubation was stopped by adding 100 µl of a stop/colour reagent, containing 2.5% (w/v) ammonium heptamolybdate, 0.25% (w/v) ammonia (25%), 0.059% (w/v) ammonium vanadate and 6 % (v/v) nitric acid (65%). The absorbance was measured at 415 nm against the blank incubation. Potassium dihydrogen phosphate was used for calibration (0-5 mM).

Example 3:

The protein fraction obtained after 50 to 70 % ammonium sulphate precipitation was fractionated by an anion exchange chromatography (Source Q, Pharmacia, Uppsala, Sweden) column of 100 ml. 100 ml of protein fraction was desalted to the start elution buffer A (20 mM Tris/HCl buffer, pH 8.0) which was carried out by dialysing against the start buffer A. Subsequently, the dialysed fraction was centrifuged at 20,000 g for 20 minutes and the supernatant was diluted to 500 ml with start elution buffer A and applied to the column. Protein was eluted with 1000 ml of elution buffer A and 2000 ml of elution buffer A and buffer B (= buffer A + 1 M NaCl) in a gradient from 0 to 35 % buffer B. Protein was detected by an UV detector at A280 nm and collected in 12.5 ml fractions. The phytase activity was measured in the collected fractions as described in Example 2. Figure 1 shows the results, two main peaks with phytase activity can be distinguished. The first main peak was pooled and the specific activity was 21 µmol/min/g protein.

Example 4:

The first main peak pooled after the anion exchange chromatography was separated by a cation exchange chromatography (Source S, Pharmacia, Uppsala, Sweden) column of 20 ml. The fraction pooled was desalted by a P6 column (Bio-Rad) and applied to the column by a 150 ml superloop. Proteins were eluted with 130 ml of elution buffer A (20 mM acetic acid/NaOH, pH 4.6) and 420 ml of elution buffer A and buffer B (= buffer A + 0.4 mM NaCl) in a gradient from 0 to 60% buffer B. Protein was detected by an UV detector at A280 nm and collected in 5 ml fractions. The results of the chromatography on Source S are shown in

Figure 2. The phytase activity was measured in the collected fractions as described in example 2. The isolated

soya phytase showed a specific activity of about 1.3 mmol/min/g of protein.

The purified fraction was analysed by SDS-PAGE (gradient 10-15%) and isoelectric focusing(IEF) with a range from pH 4 to 6.5, using the Phast system of Pharmacia according to the instructions of the manufacturer. The proteins were stained by silver staining. The results are shown in Figure 3 and 4 respectively. Figure 3 shows that the reduced and non-reduced fraction consist of one protein band and demonstrated an apparent molecular weight of about 75,000 Da. Figure 4 shows also one protein band with an isoelectric point of 4.9.

Example 5:

The purified fraction was also run on a 15% SDS-PAGE and blotted to Immobilon-P membrane in methanol/glycine transfer buffer after electrophoresis. The membrane was washed five times with distilled water, stained with Coomassie Brilliant Blue R250 in 25% (v/v) methanol and 8%(w/v) acetic acid, destained, and the main protein band was excised from the membrane and the N-terminal amino acid sequence was determined with an Applied Biosystems mode 477 A gas-phase sequencer connected on-line to a 120 A PTH Analyser. The sequencing of the N-terminus of the phytase according to the invention revealed the following sequence: H I P S T L E G P F D P V T V P F D P A L R G V A V D L P E T as given in SEQ ID NO: 1. An internal fragment of the phytase according to the invention analyzed in the corresponding manner has the following sequence: F A D E P G H X P D P L S T P D P as presented in in SEQ ID NO: 3.

These N-terminal and internal protein sequence data show no significant homology with any known protein or DNA sequences (in all possible frames).

Example 6:

Phytate (9.1 mM) and KH_2PO_4 (in a concentrations from 0 to 5 mM used for reference) were dissolved in six different buffers in the pH range from 3 to 8. Citric acid, formic acid, MES, MOPS and TAPS buffers were used for the respective pH 3,4,5,6,7 and 8. One mM $CaCl_2$ was added to each buffer and the phytase activity was determined as described above in Example 2. The isolated soya phytase demonstrated a pH optimum at pH 5 as can be seen from Figure 5.

Example 7

Based on the present disclosure the cDNA of the gene encoding the soybean phytase of the invention is cloned using a nucleic acid probe based on the amino acid sequence as given in SEQ ID No: 1, preferably using a mixture of oligonucleotides comprising the DNA sequence GARGGNCCNT TYGAYCCNGT of Seq ID NO:2 , where N is A, T, G, C or inosine, R is A or G and Y is T or C, and subsequently expressed in E. coli and soybean using procedures known in the art and described in Sambrook, J. et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, USA, (1989), Current Protocols in Molecular Biology, vol. 1, Frederick Ausubel, Ed., John Wiley and Sons, USA, and EP 301 749.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Finnfeeds International, Ltd.
(B) STREET: P.O. Box 777
(C) CITY: Marlborough
(D) STATE: Wiltshire
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): SN8 1XN

(ii) TITLE OF INVENTION: Phytase obtainable from germinated soybeans

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Soybean

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
His Ile Pro Ser Thr Leu Glu Gly Pro Phe Asp Pro Val Thr Val Pro
1               5                  10                  15

Phe Asp Pro Ala Leu Arg Gly Val Ala Val Asp Leu Pro Glu Thr
            20                  25                  30
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:6
(D) OTHER INFORMATION:/note= "N is A, T, G, C or inosine"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:9
(D) OTHER INFORMATION:/note= "N is A, T, G, C or inosine"

(ix) FEATURE:
(A) NAME/KEY: modified_base
(B) LOCATION:18
(D) OTHER INFORMATION:/note= "N is A, T, G, C or inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAGGNCCNT TYGAYCCNGT

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Soybean

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Phe Ala Asp Glu Pro Gly His Xaa Pro Asp Pro Leu Ser Thr Pro Asp
1 5 10 15

Pro

CLAIMS

1. Soybean phytase, characterised in that said phytase has an optimal pH of about 5.0, when measured in a buffer comprising 0.0091 M sodium phytate in 50 mM acetic acid/NaOH and 1 mM $CaCl_2$ at 50°C for 4 hrs.

2. Soybean phytase according to claim 1, characterised in that said phytase has a specific activity of at least 6 μmol/min/g protein when measured in a buffer comprising 0.0091 M sodium phytate in 50 mM acetic acid/NaOH and 1 mM $CaCl_2$ at 50°C for 4 hrs at pH 5.0.

3. Soybean phytase according to claim 1 or 2, characterised in that said phytase has a pI of about 4.9.

4. Soybean phytase according to any of claims 1 to 3, characterised in that said phytase has a molecular weight of between 30,000 and 100,000 Daltons, preferably 75,000 Daltons.

5. Soybean phytase according to any of claims 1 to 4, characterised in that said phytase comprises an amino acid sequence in the N-terminal portion of the protein having at least 85% homology to the amino acid sequence given in SEQ ID NO: 1 and/or an internal amino acid sequence having at least 85% homology to the amino acid sequence given in SEQ ID NO: 3.

6. Soybean phytase according to any of claims 1 to 5, characterised in that said phytase comprises the amino acid sequence given in SEQ ID NO: 1 in the N-terminal portion of the protein and/or the internal amino acid sequence given in SEQ ID NO: 3.

7. Purified soybean phytase according to any of claims 1 to 6, characterised in that said phytase is obtainable by a method comprising the steps of:

a) subjecting an extract of soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof to 50-70% ammonium precipitation to form a precipitate comprising said phytase;

b) subjecting said precipitate to anion exchange chromatography and collecting fractions containing said phytase; and

c) subjecting said precipitate to cation exchange chromatography and collecting fractions containing said phytase.

8. A DNA molecule encoding a phytase according to any of claims 1 to 7.

9. A vector DNA molecule comprising a DNA molecule according to claim 8.

10. A prokaryotic or eukaryotic organism or host cell transformed with a DNA molecule according to claim 8 or 9 and capable of expressing said phytase.

11. A prokaryotic host cell according to claim 10, characterized in that said host cell is selected from the group comprising E. coli, Bacillus sp., Lactobacillus sp. and Lactococcus sp.

12. A eukaryotic organism according to claim 10, characterized in that said organism is a fungus selected from the group comprising Aspergillus, Trichoderma, Penicillium, Mucor, Kluyveromyces and Saccharomyces or is a plant selected from the group comprising soybean, corn and rapeseed or seeds thereof.

13. Method for producing phytase, characterized in that said phytase is isolated from a prokaryotic or eukaryotic host cell or organism according to any of claims 10 to 12.

14. Method of producing phytase comprising the steps of:
a) subjecting an extract of soybeans or fractions thereof, soya flour, germinated soybeans or parts thereof to 50-70% ammonium precipitation to form a precipitate;
b) subjecting said precipitate to anion exchange chromatography and collecting fractions containing said phytase; and
c) subjecting said precipitate to cation exchange chromatography and collecting fractions containing said phytase.

15. Use of the phytase according to any of claims 1 to 7 in seeds, foods or animal feeds or fractions thereof.

16. Use of the phytase according to any of claims 1 to 7 for the treatment of cereals, legumes and other agro-materials for the preparation of food materials, feedstuffs or fractions thereof.

17. Use of a plant or seeds according to claim 12 in foods or animal feeds or fractions thereof.

18. Use according to claim 15 to 17 wherein said use reduces the environmental impact of phosphorous from livestock production.

19. Use according to claim 15 to 17 wherein said use reduces the multivalent metal ion binding antinutritional effect of phytate.

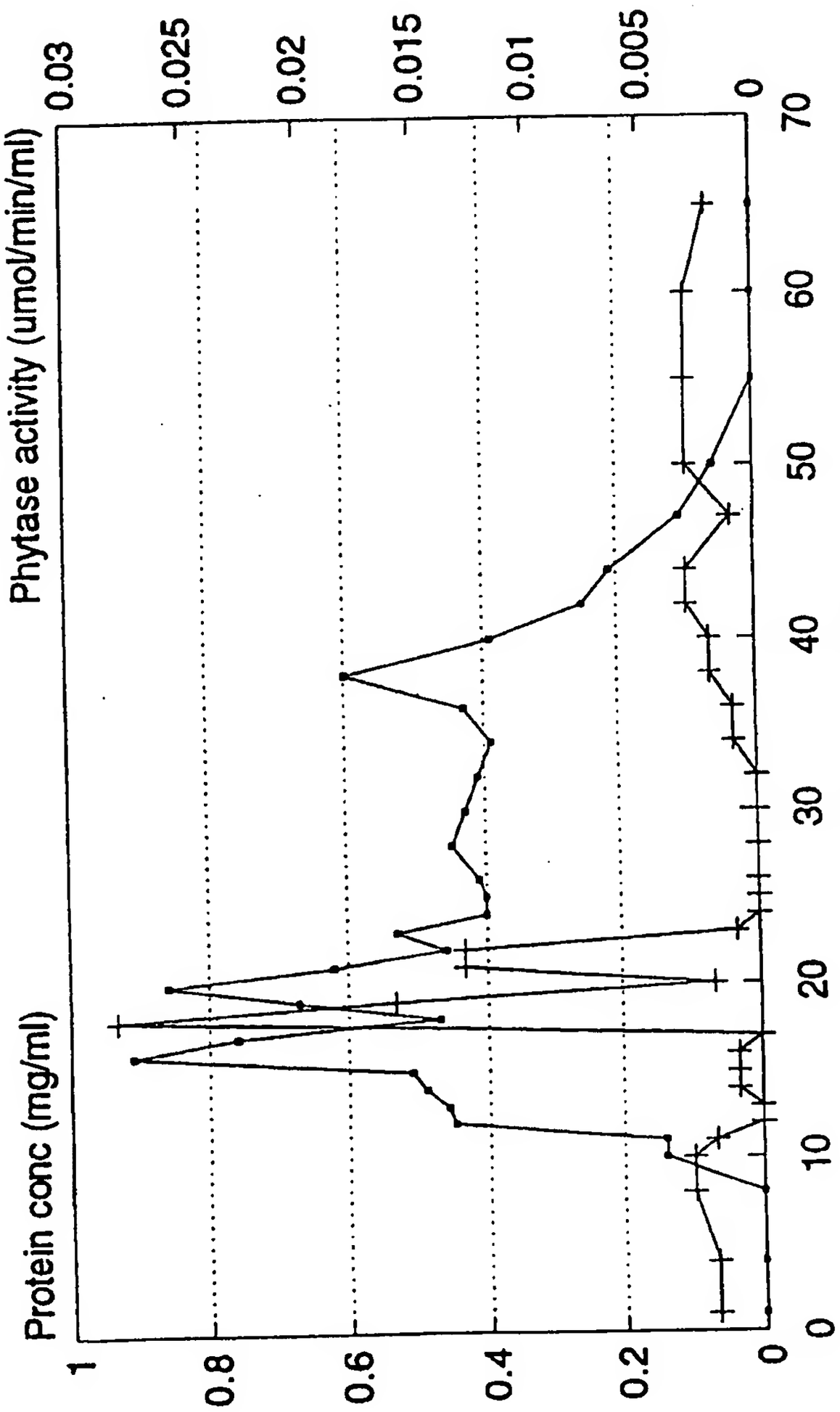

Protein conc (mg/ml)
1
0.8
0.6
0.4
0.2
0
Phytase activity (umol/min/ml)
0.03
0.025
0.02
0.015
0.01
0.005
0
70
60
50
40
30
20
10
0
Fraction number
Protein content
Phytase activity

Fig. 1

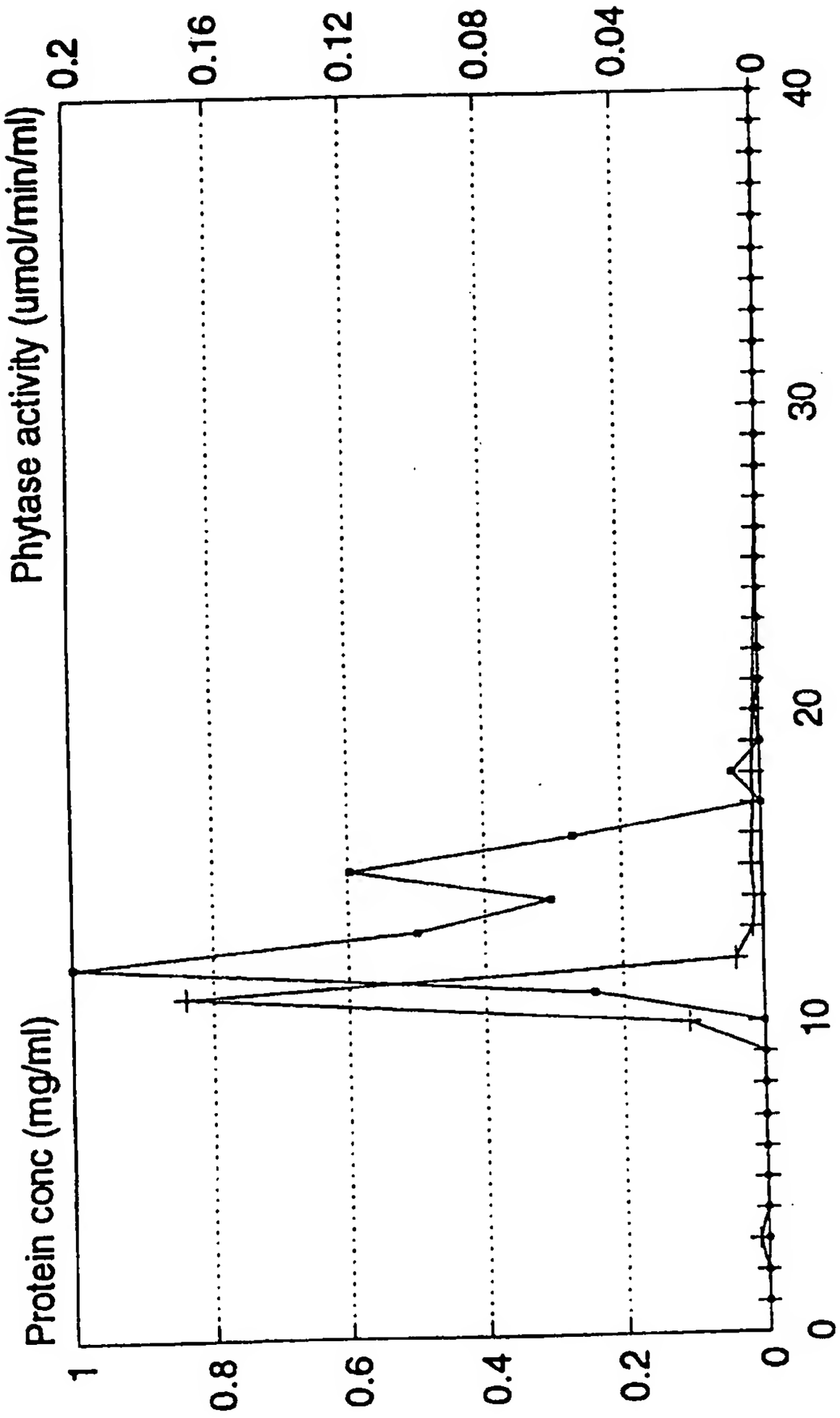

Protein conc (mg/ml)
1
0.8
0.6
0.4
0.2
0
Phytase activity (umol/min/ml)
0.2
0.16
0.12
0.08
0.04
0
40
30
20
10
0
Fraction number
Protein content
Phytase activity

Fig. 2

Fig. 3

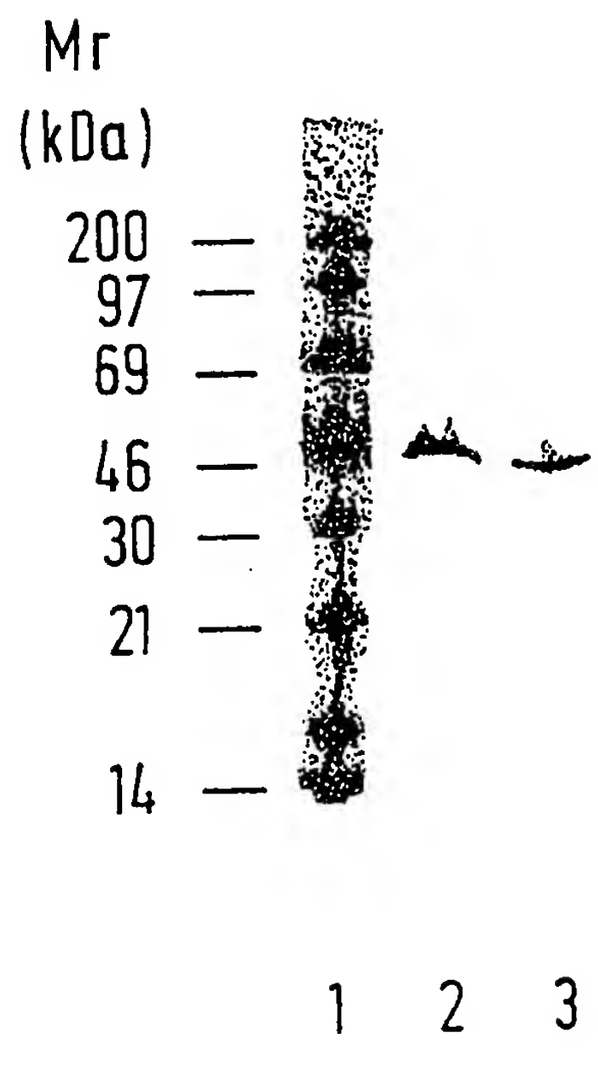


Fig. 4

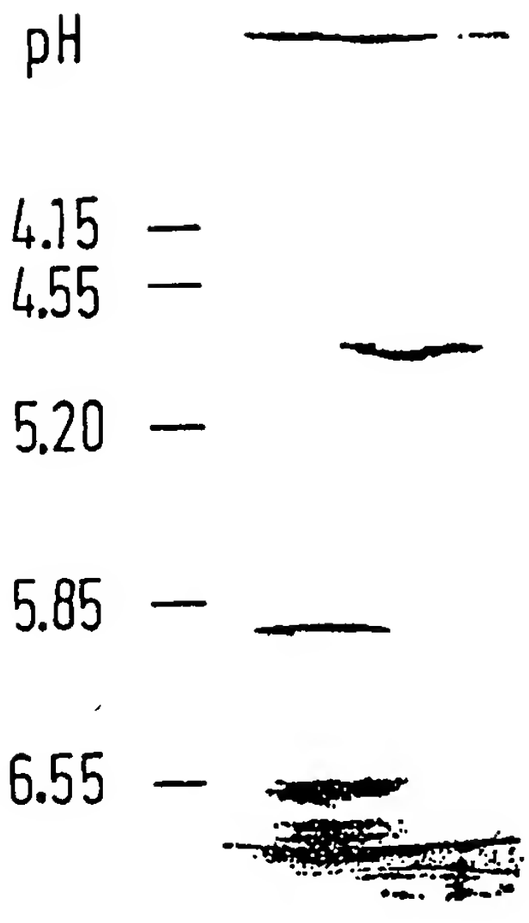
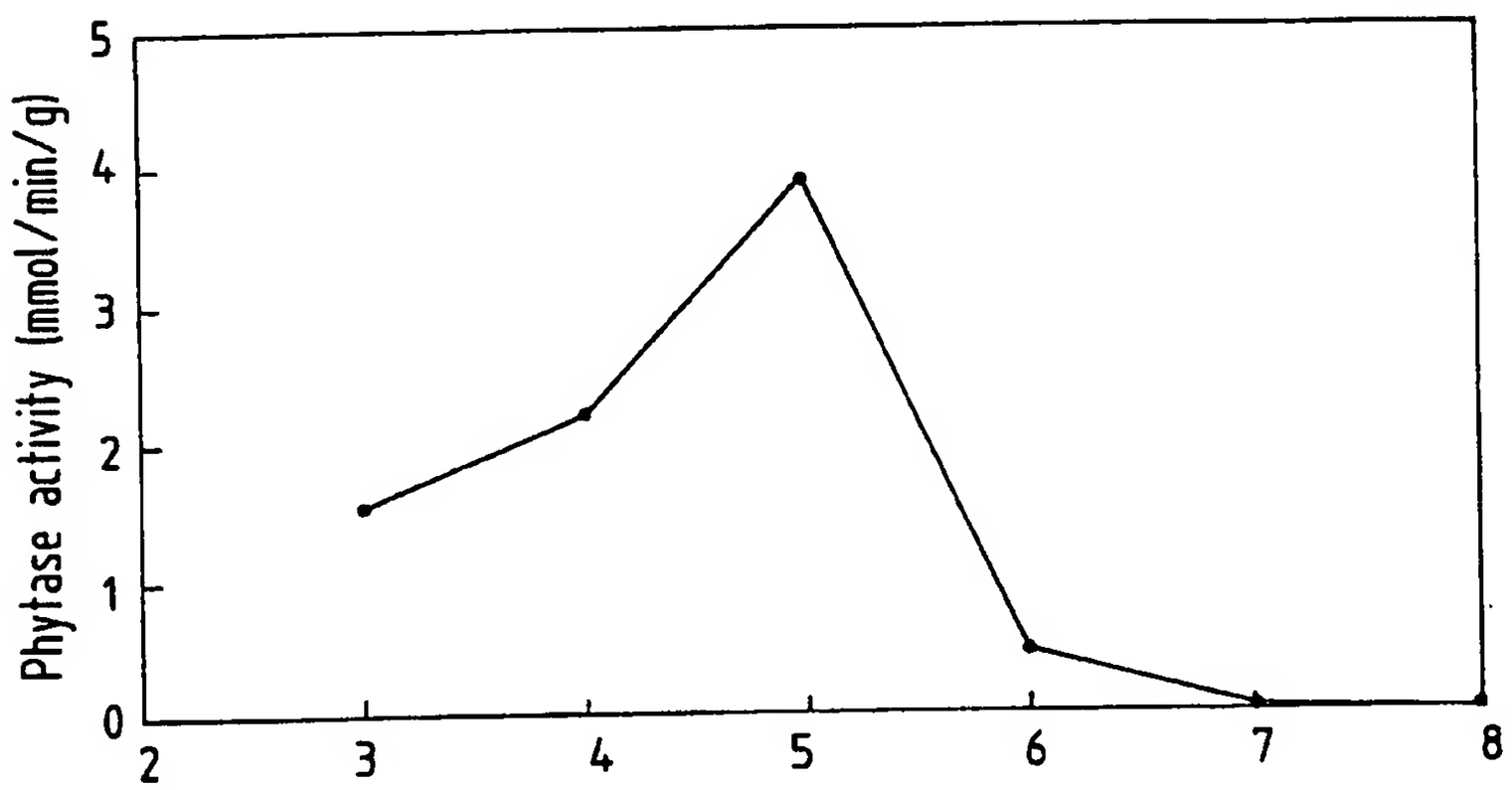


Fig. 5



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C12N 15/55, 9/16, 1/21, 1/15, 1/19, A01H 5/00, C12N 15/82, A23K 1/165, A23L 1/03, C12Q 1/42 // (C12N 1/21, C12R 1:01, 1:07, 1:19, 1:225) (C12N 1/15, C12R 1:66, 1:785, 1:80, 1:885) (C12N 1/19, C12R 1:85)

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(57) Abstract

The invention relates to a class of phytate–degrading enzymes which are endogenously present in soya flour, soybeans, germinated soybeans or fractions thereof, to a method for obtaining such enzymes as well as to the use of these enzymes in feed and food applications.

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Inter onal Application No
PCT/EP 97/06076

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N9/16 C12N1/21 C12N1/15 C12N1/19
A01H5/00 C12N15/82 A23K1/165 A23L1/03 C12Q1/42
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IPC 6 C12N A01H A23K A23L C12Q

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y		15,16, 18,19

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INTERNATIONAL SEARCH REPORT

Inter. onal Application No
PCT/EP 97/06076

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 1:80,1:885),(C12N1/19,C12R1:85)

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